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Susceptibility Testing of *Dientamoeba fragilis* ATCC 30948 with Iodoquinol, Paromomycin, Tetracycline, and Metronidazole

FRANCIS T. H. CHAN,[1]* MING XU GUAN,[1]† ANDREW M. R. MACKENZIE,[2] AND FRANCISCO DIAZ-MITOMA[1]

Department of Laboratory Medicine, Children's Hospital of Eastern Ontario, Ottawa K1H 8L1,[1] and the Division of Microbiology, Ottawa Civic Hospital, Ottawa, Ontario K1Y 4E9,[2] Canada

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Susceptibility testing was performed on *Dientamoeba fragilis* ATCC 30948 in a dixenic culture with *Klebsiella pneumoniae* and *Bacteroides vulgatus*. *D. fragilis* was cocultured with the bacteria in TYGM-9 medium (ATCC medium 1171). The activities of antiparasitic drugs were assessed by counting viable *D. fragilis* trophozoites with a hemacytometer by trypan blue exclusion. The minimal amebicidal concentrations of the following four drugs were determined: iodoquinol at 128 μg/ml, paromomycin at 16 μg/ml, tetracycline (questionably) at 32 μg/ml, and metronidazole at 32 μg/ml.

Dientamoeba fragilis is a protozoan parasite described and named by Jepps and Dobell in 1918 (9). It resides in the human large intestine. The organism is now classified as a flagellate (12), but it has no demonstrable flagella or a cyst stage. The distribution of this organism is worldwide, with reported incidences of 1.4 to 53% (3, 4, 11, 14, 17, 19, 22).

Although often considered harmless, *D. fragilis* has been associated with a variety of symptoms. Abdominal pain, diarrhea, abnormal stools, loss of appetite, and weight loss are the common symptoms that have been described previously (22). Wenrich et al. (21) reported that there is a higher incidence of gastrointestinal disorders among college students harboring *D. fragilis* than among those infected with *Entamoeba histolytica*. Sapero (15) found that 27.3% of the patients infected with *D. fragilis* had symptoms, and Steinitz et al. (18) reported symptoms in 15.1% of infected individuals. In Canada, an incidence of 25% symptomatic cases was reported by Yang and Scholten (22). However, the pathogenicity of *D. fragilis* has been questioned, mainly because studies of its prevalence and associated symptoms were uncontrolled and they suffered from selection bias. Randomized, double-blind controlled studies are needed to determine the pathogenic roles of this parasite.

In a retrospective study of "pure" *D. fragilis* infections, Spencer et al. (17) found gastrointestinal symptoms in 32 (91%) of 35 children. It was noted that diarrhea was the most common finding in patients with acute illness, whereas abdominal pain was more common in children with chronic symptoms. Treatment with iodoquinol or metronidazole was effective. Similar findings were also reported by the same group of investigators (16) for an adult population. Drugs recommended for use in the treatment of *D. fragilis* infections are iodoquinol, paromomycin, or tetracycline (13). The objective of the study described here was to determine the in vitro susceptibility of *D. fragilis* to iodoquinol, paromomycin, tetracycline, and metronidazole.

A dixenic culture of *D. fragilis* ATCC 30948 was acquired from the American Type Culture Collection (ATCC). The two bacteria listed by ATCC in this dixenic culture are *Klebsiella pneumoniae* and *Clostridium perfringens*. While the presence of *K. pneumoniae* was evident and confirmed, we were unable to isolate *C. perfringens* from the culture that we received. Instead, a *Bacteroides* sp. was isolated. The organism was identified as *Bacteroides vulgatus* by the Laboratory Centre for Disease Control, Ottawa, Ontario, Canada. The discrepancy in bacterial identification was discussed with the staff at ATCC, who indicated that they would further investigate this dixenic culture (5). The bacterial species that were cocultured helped to maintain *D. fragilis* on continuous passage in TYGM-9 medium (ATCC medium 1171).

The following antiparasitic compounds were kindly provided for our study: iodoquinol (Searle Canada Inc., Oakville, Ontario, Canada), paromomycin (Parke-Davis, Ann Arbor, Mich.), tetracycline (Pfizer Canada Inc., Arnprior, Ontario, Canada), and metronidazole (Abbot Laboratories Ltd., Montreal, Quebec, Canada). Stock solutions (10,000 μg/ml) were made in sterile distilled water (except for iodoquinol, which was prepared as a suspension in 10% ethanol because of its poor solubility in water), further diluted, and added to tubes of rice starch-supplemented culture medium adjusted to contain a volume of 8 ml. We determined in a separate experiment that the amount of ethanol used in the drug dilutions did not have deleterious effects on *D. fragilis*. Serial doubling dilutions of each drug were prepared in these tubes of medium to cover a range of 256 to 1 μg/ml. A tube of supplemented medium with no drug was included as a growth control. The *D. fragilis* inoculum was adjusted to 10^5 trophozoites in 0.1 ml by low-speed centrifugation (100 × *g* for 10 min) and counting with a hemacytometer. After seeding with 0.1 ml of inoculum containing *D. fragilis* trophozoites and the two bacteria, all inoculated tubes were incubated at 35°C. The number of live trophozoites in each tube was determined by trypan blue exclusion by taking the average of three counts on a daily basis. Growth of *D. fragilis* in the control tube peaked to 7.7×10^5 trophozoites per ml in three days (Table 1), after which the number of live trophozoites declined. The daily counting was therefore continued for 3 days for the drug-containing tubes of medium.

Table 1 shows the activities of the four drugs against *D. fragilis*. It was a common finding for all four drugs that the number of viable *D. fragilis* trophozoites decreased as the concentration of the drugs increased. Viable trophozoites also disappeared abruptly at a particular concentration of each drug. At and above this amebicidal concentration, viable *D.*

* Corresponding author.
† Present address: Veterinary Science, University of Kentucky, Lexington, KY 40546-0099.

TABLE 1. Minimal amebicidal concentrations[a] of iodoquinol, paromomycin, tetracycline, and metronidazole for *D. fragilis* ATCC 30948 determined by viable counts performed daily over 3 days

Drug concn (μg/ml)	Viable counts (10^4/ml) over 3 days											
	Iodoquinol			Paromomycin			Tetracycline			Metronidazole		
	1 day	2 days	3 days	1 day	2 days	3 days	1 day	2 days	3 days	1 day	2 days	3 days
0[b]	10	43	77									
1	10	33	62	7.5	18	33	12	25	53	10	32	66
2	9.7	32	49	7.5	15	25	9.0	31	48	11	26	56
4	8.5	28	53	7.5	15	25	7.0	27	43	9.8	19	45
8	6.5	23	43	7.5	15	18	6.0	20	40	5.7	9.8	33
16	6.2	19	40	0	0	0	3.8	20	37	3.0	1.8	32
32	5.5	15	37	0	0	0	0	0	0	0	0	0
64	4.7	10	30	0	0	0	0	0	0	0	0	0
128	0	0	0	0	0	0	0	0	0	0	0	0
256	0	0	0	0	0	0	0	0	0	0	0	0

[a] Iodoquinol, 128 μg/ml; paromomycin, 16 μg/ml; tetracycline, 32 μg/ml (see text), and metronidazole, 32 μg/ml.
[b] Growth control tube with no drugs.

fragilis trophozoites were not found. This "all-or-none" phenomenon was probably due to the inability of the fragile trophozoites to repair and revive themselves in the presence of the antiparasitic drugs, and the damage became irreversible. From a practical point of view, it was also difficult to find very small numbers of viable trophozoites microscopically with the small volumes used in a hemacytometer for counting. The minimal amebicidal concentrations of the following drugs were determined: iodoquinol at 128 μg/ml, paromomycin at 16 μg/ml, tetracycline at 32 μg/ml, and metronidazole at 32 μg/ml (Table 1).

Because these antiparasitic drugs could also be antibacterial, we performed agar dilution testing on *K. pneumoniae* and *B. vulgatus*, the two supporting bacteria in the *D. fragilis* culture. Serial doubling dilutions of the four drugs were prepared in Mueller-Hinton agar (Becton Dickinson Microbiology Systems, Cockeysville, Md.) with 5% defibrinated sheep blood. On the basis of the amebicidal results obtained with *D. fragilis*, the following ranges of serial doubling dilutions of the four drugs were prepared in the agar dilution test: 128 to 1 μg/ml for iodoquinol, 16 to 1 μg/ml for paromomycin, 32 to 1 μg/ml for tetracycline, and 32 to 1 μg/ml for metronidazole.

While the *K. pneumoniae* strain in the dixenic culture appeared to be uniform in colonial morphology, *B. vulgatus* presented consistently with two different colony sizes which we designated B1 (small colonies) and B2 (large colonies). The biochemical reactions of variants B1 and B2 were similar (data not shown). We include here susceptibility studies on both the B1 and B2 variants of *B. vulgatus*.

Fresh (24-h) cultures of *K. pneumoniae* as well as the B1 and B2 variants of *B. vulgatus* were suspended in 5 ml of Mueller-Hinton broth (Oxoid Limited, Basingstoke, England) to match a 0.5 McFarland turbidity standard (approximately 10^8 CFU/ml). These bacterial suspensions were further diluted 1 in 10 to give a concentration of 10^7 CFU/ml. A 1-μl volume of this diluted suspension was inoculated onto each plate of the drug dilution series. A control plate with no drugs was included as a growth control. All the inoculated plates were incubated anaerobically at 35°C for 48 h.

The MICs of the four drugs for the bacteria are given in Table 2. There was no inhibition of *K. pneumoniae* or *B. vulgatus* (both B1 and B2 variants) by iodoquinol (highest concentration tested, 128 μg/ml) or paromomycin (highest concentration tested, 16 μg/ml). The *K. pneumoniae* strain was highly susceptible to tetracycline, (MIC, ≤1 μg/ml). There was a twofold difference in the tetracycline MICs for the two *B. vulgatus* variants: 16 μg/ml for variant B1 and 32 μg/ml for variant B2. Metronidazole up to a concentration of 32 μg/ml did not exhibit any inhibitory activity against *K. pneumoniae*, while both *B. vulgatus* variants were inhibited by metronidazole at a concentration of ≤1 μg/ml.

By comparing the activities of the four drugs against *D. fragilis* in the dixenic culture and the bacterial isolates, we were led to the following conclusions. Because of the lack of antibacterial activity by iodoquinol and paromomycin under the test conditions of the present study, the amebicidal activity exerted against *D. fragilis* should be directly related to these two drugs. On the other hand, the activity of tetracycline against *D. fragilis* was difficult to assess. The *K. pneumoniae* strain was highly susceptible to tetracycline (≤1 μg/ml), and both variants of *B. vulgatus* were inhibited by tetracycline at a concentration of 32 μg/ml, the same concentration at which depletion of live *D. fragilis* trophozoites was observed. The inhibition of growth of *D. fragilis* by tetracycline might be attributed to this concentration of the drug or it may be a result of the depletion of the bacteria which supported the growth of the trophozoites. Metronidazole was highly active against both *B. vulgatus* variants (MIC, ≤1 μg/ml), but it had no activity against *K. pneumoniae*. This can be expected because the cytotoxic selectivity of metronidazole results from the necessity of the drug to be reduced to an active cytotoxic form, and this does not occur in aerobic cells (10). It appears that under the action of metronidazole the growth of *D. fragilis* was sustained by *K. pneumoniae* as a monoxenic culture and that the MIC of 32 μg/ml (versus ≤1 μg/ml for *B. vulgatus*) was a "true" MIC for *D. fragilis*.

To summarize, we performed antiparasitic drug susceptibility testing on the ATCC dixenic culture of *D. fragilis*. With the

TABLE 2. MICs of iodoquinol, paromomycin, tetracycline, and metronidazole for *K. pneumoniae* and two variants of *B. vulgatus*

Organism	MIC (μg/ml)			
	Iodoquinol	Paromomycin	Tetracycline	Metronidazole
K. pneumoniae	>128	>16	≤1	>32
B. vulgatus variant B1	>128	>16	16	≤1
B. vulgatus variant B2	>128	>16	32	≤1

exception of tetracycline, which exhibited inhibition against both *K. pneumoniae* and *B. vulgatus*, and therefore making the results of the study on *D. fragilis* inconclusive, all other drugs (iodoquinol, paromomycin, and metronidazole) yielded results which could indicate that they were inhibitory against *D. fragilis* because of their limited or total lack of activity against the supporting bacteria. However, we do not know the complexities and influences of the bacteria in the dixenic culture. It is possible that subinhibitory concentrations of antimicrobial agents could affect the bacterial populations in more subtle ways, such as causing the release of toxins or growth factors that might have an adverse effect on the viability of the parasite. We tried to avoid performing susceptibility testing on *D. fragilis* in a mixed bacterial culture through axenization with imipenem and piperacillin to eliminate the bacteria (5). Unfortunately, attempts to axenize *D. fragilis* by this and other means, including increasing the concentration of bovine serum to 15% (7), using a *Crithidium* sp. strain, ATCC 50083 (6), adding preconditioned medium obtained from bacterial growth, adding heat-killed bacteria, and incubating the cultures under anaerobic conditions (23), have failed. Perhaps *D. fragilis* can be axenized by eliminating the anaerobes first by repeated passage in the presence of low concentrations of metronidazole. Then it may be possible to eliminate *K. pneumoniae* with low concentrations of tetracycline or other antibiotics. We also tried to perform drug testing by the agar colony method (8), but the necessity of maintaining bacterial growth for sustaining the viability of *D. fragilis* made it impossible to quantify *D. fragilis* by this technique. In the absence of an axenic culture, we could achieve drug susceptibility testing results for *D. fragilis* only in the presence of the supporting bacteria. We noticed that earlier studies on drug testing with *D. fragilis* and other intestinal parasites were performed by Balamuth (1) with bacterial cultures. We also believe that antiparasitic drug testing in the presence of bacteria is probably clinically relevant because it may simulate the in vivo situation.

There have been few studies on *D. fragilis*, even though the parasite was described more than 70 years ago, and no susceptibility studies of the drugs mentioned have been done in the laboratory. Iodoquinol, paromomycin, and tetracycline are listed as the drugs of choice for the treatment of *D. fragilis* infections (13), but we were unable to find experimental data to substantiate this recommendation. Metronidazole is not recommended as a drug for the treatment of *D. fragilis* infections, but it was described by Spencer et al. (17) as an effective agent. However, the drug's mutagenicity and carcinogenic potential make it a less than ideal drug for use in children (20). As with the other three drugs tested, experimental details on the susceptibility testing of metronidazole against *D. fragilis* are also lacking.

We demonstrated in the present study that, in the absence of an axenic culture, an in vitro drug susceptibility test can be performed on *D. fragilis* in the presence of growth-supporting bacteria. The minimal amebicidal concentrations on *D. fragilis* determined in our study were 128 μg/ml for iodoquinol, 16 μg/ml for paromomycin, 32 μg/ml (questionably) for tetracycline, and 32 μg/ml for metronidazole. It is difficult to correlate these minimal amebicidal concentrations with the clinical responses reported in the literature because of the lack of knowledge on the levels of the antimicrobial agents in the gut. There is virtually no information on the concentrations of iodoquinol in the gastrointestinal tract, although it is reckoned that only about 5% of the drug is absorbed from the gastrointestinal tract (15a). Paromomycin is poorly absorbed from the gastrointestinal tract, and most of an oral dose is excreted unchanged in the feces. Unfortunately, there are no data on the levels of paromomycin in the gastrointestinal tract (14a). Tetracycline is a well-established antimicrobial agent, but a search of the literature published over the past 30 years did not reveal any information on the concentration of the drug in the gut (14b). In a study on metronidazole therapy for antibiotic-associated colitis caused by *Clostridium difficile*, Bolton and Culshaw (2) reported a maximal metronidazole concentration in feces of 24.2 μg/g and a maximal hydroxy-metronidazole concentration in feces of 62.4 μg/g. This may explain the reported effective treatment of *D. fragilis* infections with metronidazole (17). While our study was performed on the ATCC strain of *D. fragilis* (which originated from a patient), the same procedure can be applied to a clinical isolate if it can be established in culture. Unfortunately, it is difficult to establish *D. fragilis* isolates from patients in culture, and this may limit the application of the assay in the clinical management of *D. fragilis* infections. In the absence of a more refined protocol for susceptibility testing of *D. fragilis*, the approach described here can probably be used to test newer drugs as well, if they become available. If we consider the prevalence and pathogenic potential of *D. fragilis*, it is amazing to see that so little progress has been made on the study of this organism and that there is such a lack of experimental data on drug interaction with *D. fragilis*. As pointed out by Balamuth (1) in 1953: "There is no question that a standardized technique is strongly needed and long overdue in the interest of accuracy and repeatability of claims. This advance has been impeded by our present inability to maintain these parasites in pure culture as well as by ignorance of other requirements existing in their complex environment." This situation has not changed over the past 40 years for *D. fragilis*. It should be cautioned, however, that the susceptibility testing results reported here were obtained on a single ATCC isolate, and it remains to be demonstrated how generalizable these results will be. Research toward a better understanding of the epidemiology, pathogenicity, diagnosis, and treatment of this interesting organism is very much needed.

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